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Enzymatic hydrolysis of cellulose by the cellobiohydrolase domain of CelB from the hyperthermophilic bacterium *Caldicellulosiruptor saccharolyticus*

Joshua I. Park [a,b], Michael S. Kent [a,d], Supratim Datta [a,b], Bradley M. Holmes [a,b], Zhaohua Huang [e], Blake A. Simmons [a,b], Kenneth L. Sale [a,c], Rajat Sapra [a,b,*]

[a] *Deconstruction Division, Joint BioEnergy Institute, Emeryville, CA 94608, USA*
[b] *Department of Biomass Science and Conversion Technology, Sandia National Laboratories, Livermore, CA 94551, USA*
[c] *Biosystems R&D Department, Sandia National Laboratories, Livermore, CA 94551, USA*
[d] *Sandia National Laboratories, Albuquerque, NM 87123, USA*
[e] *Departments of Bioengineering, Therapeutic Sciences, and Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA 94143, USA*

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ABSTRACT

The *celB* gene of *Caldicellulosiruptor saccharolyticus* was cloned and expressed in *Escherichia coli* to create a recombinant biocatalyst for hydrolyzing lignocellulosic biomass at high temperature. The GH5 domain of CelB hydrolyzed 4-nitrophenyl-β-D-cellobioside and carboxymethyl cellulose with optimum activity at pH 4.7–5.5 and 80 °C. The recombinant GH5 and CBM3-GH5 constructs were both stable at 80 °C with half-lives of 23 h and 39 h, respectively, and retained >94% activity after 48 h at 70 °C. Enzymatic hydrolysis of corn stover and cellulose pretreated with the ionic liquid 1-ethyl-3-methylimidazolium acetate showed that GH5 and CBM3-GH5 primarily produce cellobiose, with product yields for CBM3-GH5 being 1.2- to 2-fold higher than those for GH5. Confocal microscopy of bound protein on cellulose confirmed tighter binding of CBM3-GH5 to cellulose than GH5, indicating that the enhancement of enzymatic activity on solid substrates may be due to the substrate binding activity of CBM3 domain.

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1. Introduction

Recently there has been renewed interest liberating fermentable sugars from pretreated lignocellulosic biomass by utilizing mixtures of enzymes that hydrolyze biomass for ethanol and other biofuels production (Banerjee et al., 2010; Gao et al., 2010). Employing active biomass hydrolyzing enzymes at extremes of temperature and pH may be advantageous for industrial scale production of fermentable sugars from lignocellulosic biomass, because these conditions facilitate overcoming biomass recalcitrance and prevent the growth of contaminating microorganisms (Blumer-Schuette et al., 2008). Cellulases, which include endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (3.2.1.21), are members of the glycoside hydrolase (GH) families of enzymes that catalyze the cleavage of β-1,4-glycosidic bonds of cellulose to glucose (Henrissat and Davies, 1997; Lynd et al., 2002). In nature, these enzymes are found in microorganisms that thrive in extreme environments (Sunna et al., 1997; Bergquist et al., 1999; Blumer-Schuette et al., 2008); therefore, cellulases from extremophiles are attractive as potential biocatalysts for industrial depolymerization of cellulose.

Caldicellulosiruptor saccharolyticus (*C. sac*) is a thermophilic anaerobic bacterium first isolated from thermal springs in New Zealand (Sissons et al., 1987). *C. sac* is capable of growing on cellulose as its sole carbon source at an optimum growth temperature of 70 °C, presumably by secreting the cellulolytic enzymes required to produce glucose for survival and growth (Rainey et al., 1994). Recently, it was reported that *C. sac* also grows on insoluble switchgrass at 70 °C without the need for any other carbon sources (Yang et al., 2009). Genomic analysis of *C. sac* revealed the presence of several gene clusters encoding multi-domain hydrolases, including cellulases and xylanases (Gibbs et al., 2000; van de Werken et al., 2008). To date, both *celA* and *celB* from *C. sac* have been confirmed as bi-functional cellulolytic genes (Saul et al., 1990; Te'o et al., 1995). However, detailed biochemical studies have not been reported for either recombinant endoglucanases or cellobiohydrolases from *C. sac*, presumably due to the difficulty of producing sufficient quantities of recombinant proteins. Previously, Saul and

Abbreviations: [C2mim][OAc], 1-ethyl-3-methylimidazolium acetate; *C. sac*, *Caldicellulosiruptor saccharolyticus*; CBM, carbohydrate binding module; CMC, carboxymethyl cellulose; CV, column volume; GH, glycoside hydrolase; *p*NPC, 4-nitrophenyl-β-D-cellobioside; *p*NPG, 4-nitrophenyl-β-D-glucopyranoside.

* Corresponding author. Address: 5885 Hollis Street 4th Floor, Emeryville, California 94608, USA. Tel.: +1 510 508 9956; fax: +1 510 486 4252.
E-mail addresses: rajatsapra@gmail.com, rsapra@lbl.gov (R. Sapra).

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coworkers reported that *C. sac* has a multi-domain gene, *celB*, containing a carbohydrate binding module (CBM3) flanked by two putative GH10 and GH5 domains in a 3120 bp-long open reading frame (Saul et al., 1990). Based on the enzyme activities of truncated *celB* constructs on soluble model substrates, CelB was annotated as a bi-functional cellulase that has both cellobiohydrolase (GH10) and endoglucanase (GH5) activities (Saul et al., 1990). The *celB* gene also contains an N-terminal putative signal peptide sequence (amino acid residues 1–36) without a putative transmembrane domain, which suggests that CelB is an extracellular enzyme excreted to hydrolyze cellulosic materials outside of the cell membrane. Recent proteomic analysis of the *C. sac* secretome identified CelB as one of the two extracellular cellulases (Muddiman et al., 2010); therefore, CelB may be a key extracellular enzyme for degradation of polysaccharides for survival and growth of *C. sac*.

Since the earlier study of CelB showed enzymatic activity at high temperature (Saul et al., 1990), expression and purification of the functional domain of CelB were pursued with the codon-optimized gene to create a recombinant biocatalyst. A total of five recombinant CelB constructs consisting of combinations of the three functional domains (GH10-CBM3-GH5, GH10-CBM3, CBM3-GH5, GH10, and GH5) were expressed in *Escherichia coli*, but only CBM3-GH5 and GH5 expressed as soluble proteins. Therefore, this study was aimed at investigating the influence of the CBM3 domain on the thermostability and the enzymatic activity of the GH5 domain on CMC, microcrystalline cellulose (Avicel), Avicel pretreated with 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]), corn stover, and corn stover pretreated with [C2mim][OAc].

2. Methods

2.1. Cloning truncated celB constructs for protein expression in E. coli

The amino acid sequence of CelB from *C. sac* (UniProt ID: A4XIF7) containing GH10, CBM3, and GH5 domains was obtained from the UniProt database (www.uniprot.org). Based on the amino acid sequence of CelB, the codon-optimized gene was synthesized for protein expression in *E. coli* (GenScript, Piscataway, NJ; Supplementary Fig. 1). The domain boundaries in the *celB* gene were defined by Pfam annotations (http://pfam.sanger.ac.uk) to design PCR primers for creating truncated constructs of *celB* (GH10-CBM3-GH5, GH10-CBM3, CBM3-GH5, GH10, and GH5). All PCR primers included the attB1 and attB2 recombination sites for Gateway cloning (Invitrogen, Carlsbad, CA). The amplicons were inserted into the pDONR221 plasmid to create entry clones (Supplementary Fig. 2). The entry clones in the pDONR221 vector were then recombined with the pET DEST42 vector to create constructs for protein expression in *E. coli*. All constructs in the pET DEST42 vector contained the nucleotide sequence for the c-terminal V5 epitope and His (×6) tags. The nucleotide sequence of each construct was verified by DNA sequencing (Quintara, Berkeley, CA).

2.2. Protein expression and purification

Small scale protein expression of truncated *celB* in *E. coli* and cellulase activity screenings on the soluble substrate carboxymethyl-cellulose (CMC) were performed as described previously (Allgaier et al., 2010). For larger scale protein expression of recombinant CBM3-GH5 or GH5, 10 mL of overnight culture was inoculated into 1 L of fresh LB medium containing 50 μg/mL of carbenicillin. The cells were grown at 37 °C with constant shaking at 200 RPM. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG), to a final concentration of 0.5 mM, to the cell culture at an OD_{600nm} between 0.6 and 0.9, and then the cells were grown at 30 °C for overnight with constant shaking at 200 RPM. The cells containing the expressed proteins were harvested by centrifugation at 6,000 g for 15 min at 4 °C. The cell pellet was resuspended in 20 mL of lysis buffer (300 mM NaCl, 20 mM Tris–Cl, 20 mM imidazole, pH 8, 0.2% Triton X-100, and 1 mM phenylmethylsulfonylfluoride) per L culture, and then stored at −80 °C until the frozen cell pellet was ready for protein purification.

To purify the recombinant proteins, the frozen cell suspension was thawed at room temperature, and lysozyme (1 mg/mL) and a protease inhibitor cocktail (Calbiochem, Gibbstown, NJ) were added to lyse the cells. The cell suspension was then incubated on ice for 30 min. Then the cell lysate was sonicated with three cycles of 30 s-pulse. The nucleic acids in the cell lysate suspension were digested by adding Benzonase (25 U/ml; Novagen, Gibbstown, NJ) and incubating at room temperature for 30 min, and the cell lysates were heated at 70 °C for 30 min to precipitate the native heat-labile *E. coli* proteins. The soluble extract containing the recombinant CBM3-GH5 or GH5 was obtained by centrifugation at 12,000 g for 30 min at 4 °C. The soluble extract was collected, and 10 mM β-mercaptoethanol was added to the extract. The resulting soluble extract was filtered through 0.45 μm membrane and the filtrate was used for purification of the recombinant proteins. The His(×6)-tagged recombinant CBM3-GH5 or GH5 was purified using an ÄKTA Explorer (GE Healthcare Life Sciences, Piscataway, NJ). A HisTrap FF Ni^{2+}-NTA affinity column (GE Healthcare) was pre-equilibrated with a wash buffer containing 300 mM NaCl, 20 mM Tris–Cl, and 20 mM imidazole, pH 8.0 prior to loading the soluble extract onto the column. The flow-rate was held at constant rate of 1 mL/min for all subsequent steps. The column was washed with 10 column volumes (CV) of the wash buffer. A linear gradient was applied to the column with elution buffer containing 300 mM NaCl, 20 mM Tris–Cl, 500 mM imidazole, pH 8.0 over 20 CV. The eluates containing the recombinant protein were pooled, and the buffer was exchanged to 20 mM Tris–Cl, pH 8.0 using an Econo-Pac DG-10 column (Bio-Rad, Hercules, CA). The buffer exchanged protein was loaded onto a HiTrap Q HP anion-exchange column (GE Healthcare) that was pre-equilibrated with 20 mM Tris–Cl, pH 8.0. Then a linear gradient was applied to the column with 1 M NaCl, 20 mM Tris–Cl, pH 8.0 over 20 CV to elute the bound protein. The collected fractions were analyzed by SDS-PAGE. The protein concentrations were determined by Bradford assay (Bio-Rad).

2.3. Cellulase activity screening and enzyme assays

Cellulase activity of the *celB* constructs was tested by Congo red assay on CMC-agar plates (Teather and Wood, 1982). The soluble extracts from the heat-treated cell lysates, which had been incubated at 75 °C for 30 min as described above, containing expressed recombinant proteins were spotted on CMC-agar plate (0.2% CMC and 1.5% agar), and then the plate was incubated at 37 °C for 4 h. After incubation, the plate was rinsed with 70% of ethanol, and was stained by the Congo red dye solution (0.1% in water) for 1 h. Unbound dye was washed away with 1 M NaCl until the cleared-zones were visible. CMC was also used to determine the optimum pH (pH_{opt}) and temperature (T_{opt}) of CBM3-GH5 and GH5 activities. A buffer cocktail containing sodium acetate (NaOAc), 2-(*N*-morpholino) ethanesulfonic acid (MES), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was prepared for pH-dependent enzyme activity measurement as described previously (Allgaier et al., 2010). The enzyme activity of CBM3-GH5 or GH5 was measured by mixing 1 μg of enzyme and 1% CMC in 50 μL of total volume of reaction containing 100 mM NaOAc, 50 mM MES, and 50 mM HEPES at various pH. After enzyme reaction at T_{opt}, the 3,5-dinitrosalicylic acid (DNS)

assay (Miller, 1959) was performed to measure the reducing ends of CMC after enzymatic reaction. At the end of enzyme reaction, 120 μL of DNS reagent containing 1.3 M DNS, 1 M potassium sodium tartrate, and 0.4 N NaOH was added and incubated at 95 °C for 5 min to label reducing ends of hydrolyzed CMC, and absorbance was measured at 540 nm to determine the relative enzyme activities between pH 4.0 and 8.0.

To examine the substrate specificity of the GH5 domain, enzyme assays were performed with 4-nitrophenyl-β-D-cellobioside (*p*NPC) and 4-nitrophenyl-β-D-glucopyranoside (*p*NPG) (Deshpande et al., 1984). The assay mixture contained 5 mM *p*NPC or *p*NPG in 50 mM MES buffer at pH 5.5 and 10 μg of enzyme in 1 mL reaction volume. The enzymatic hydrolysis reaction was performed at 80 °C for 10–30 min, and then each reaction mixture was quenched by adding an equal volume of 2% Na_2CO_3. The absorbance of the end product was measured at 420 nm, and the concentration of the product was interpolated against a standard curve of 4-nitrophenol in the same buffer. The rate of product formation was measured using various substrate concentrations between 0.1 mM and 25 mM of *p*NPC with 10 μg of either CBM3-GH5 or GH5. Eadie–Hofstee plots were used to calculate the kinetics parameters (V_{max}, K_m, and k_{cat}) for CBM3-GH5 and GH5. Product inhibition by cellobiose on the enzyme activity of CBM3-GH5 or GH5 was measured by adding various concentration of cellobiose in the enzyme reaction mixture containing 5 mM *p*NPC and 10 μg of either CBM4-GH5 or GH5 in 1 mL of total volume.

2.4. Differential scanning calorimetry and thermostability measurements of CBM3-GH5 and GH5

Melting temperatures (T_m) of the recombinant CBM3-GH5 and GH5 were measured by Multi-Cell Differential Scanning Calorimeter (Calorimetry Sciences Corporation, Lindon, UT). The recombinant CBM3-GH5 and GH5 were transferred to stainless cells at 3 mg/mL concentration in 100 mM NaCl, 50 mM MES buffer at pH 5.5. The cells were heated from 30 to 110 °C at the rate of 0.5 °C/min, and then cooled down at the same rate from 110 to 30 °C to examine whether the thermal denaturation of the proteins was irreversible. The thermostability of the recombinant CBM3-GH5 and GH5 as a function of time was measured by incubating the purified proteins at 70, 80, or 95 °C in 50 mM MES at pH 5.5 (0.1 mg/mL protein concentration). The enzymatic activity of CBM3-GH5 and GH5 after various time of incubation were measured by *p*NPC assay at 80 °C, in the reaction mixture containing 5 mM of *p*NPC in 50 mM MES at pH 5.5. The activity of CBM3-GH5 and GH5 at each time point was normalized as the percent of enzymatic activity at time zero at each corresponding temperature. Linear regression analysis was used to calculate the half-life of CBM3-GH5 and GH5.

2.5. Enzymatic hydrolysis of Avicel and corn stover by GH5

Microcrystalline cellulose (Avicel) was obtained from Fluka (St. Louis, MO) and corn stover was obtained from Professor Bruce Dale at Michigan State University, Lansing, MI. These solid substrates were pretreated with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) to prepare amorphous cellulose and corn stover samples as described previously (Datta et al., 2010). Each reaction vial contained 50 mg of one of the four solid substrates (untreated Avicel, [C2mim][OAc]-pretreated Avicel, untreated corn stover, or [C2mim][OAc]-pretreated corn stover) and 10 μg of either CBM3-GH5 or GH5 in 50 mM MES buffer at pH 5.5. The total reaction volume was 1 mL, and the hydrolysis reaction was carried out at 80 °C. The products formed at various time points were measured by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) on a 3 × 150 mm Dionex Carbopac PA-20 analytical column (Dionex, Sunnyvale, CA). The column was pre-equilibrated with 14 mM NaOH at a constant flow rate of 0.4 mL/min, and after sample injection, this initial condition was maintained for 12 min. Then a linear gradient of 0–100% 450 mM NaOH was applied over 20 min, followed by regeneration of the column with a linear gradient of 100–0% 450 mM NaOH over 10 min. The chromatograms of samples at various time points were compared to those of cellobiose and glucose standards to identify the products. The concentration of each product was quantified by integrating the area under the curve of the individual peaks to a calibration curve of the standards (15–150 μM).

2.6. Confocal imaging of binding interactions between CBM3-GH5 or GH5 and cellulose

The proteins were labeled using the amine reactive dye Alexa-488 succinimidyl ester (Invitrogen) following procedures supplied by the manufacturer. The concentration of labeled protein was determined by UV absorption at 280 nm after correcting for the contribution of the dye measured at 495 nm. The degree of labeling was 0.9 ± 0.1 dye molecule per protein molecule as determined by absorption at 495 nm. CBM3, GH5, and CBM3-GH5 extinction coefficients were calculated from their sequences using the ExPASy Proteomic Server (http://ca.expasy.org/tools/protparam.html) and were 57,400 $M^{-1}cm^{-1}$, 181,000 $M^{-1}cm^{-1}$, and 205,000 $M^{-1}cm^{-1}$, respectively. The manufacturer reported extinction coefficient of 71,000 $M^{-1}cm^{-1}$ for the dye with the correction factor of 0.11 for the contribution of the dye to the absorbance at 280 nM (Invitrogen) was used. The concentration of the stock solutions of labeled proteins ranged from 2 to 20 μM. The stock solutions of the labeled proteins were stored in a refrigerator at 5 °C. The proteins and substrates were incubated at 70 °C for 30 min in 50 mM sodium acetate buffer pH 5.0 and then centrifuged and extensively rinsed with buffer.

Fluorescence images of cellulose particles with adsorbed enzymes were obtained using a spinning disk confocal fluorescence microscope. For each sample a minimum of eight particles were imaged at a depth (z) resolution of 1 μm and an in-plane resolution of 1.2 μm with an exposure of 300 ms. Intensities were evaluated at z values corresponding to the midplane of the particles. The images showed a band of elevated intensity around the edges of the particles. Line profiles of intensity through the edges were taken at a minimum of five positions for each particle and the maximum values were recorded. After subtracting the background intensity, the mean and standard deviation were reported for the 40 measurements for each sample condition.

3. Results and discussion

3.1. Protein expression and purification

Five truncated constructs of *celB* (GH10-CBM3-GH5, GH10-CBM3, CBM3-GH5, GH10, and GH5) were cloned into the pET DEST42 vector for protein expression in *E. coli* (Fig. 1). These constructs did not contain the putative signal peptide so as to facilitate the accumulation of over-expressed target proteins in the *E. coli* cytosol. Functional screening on CMC-agar plates using the Congo Red zone-clearing assay revealed that only the constructs containing the GH5 domain (GH10-CBM3-GH5, CBM3-GH5, and GH5) hydrolyzed CMC. In spite of efforts to optimize protein expression of CelB in *E. coli*, the constructs containing the GH10 domain sequence produced mostly insoluble proteins. Therefore, this study was focused on functional characterizations on the GH5 domain with and without the CBM3 domain. GH5 and CBM3-GH5 were

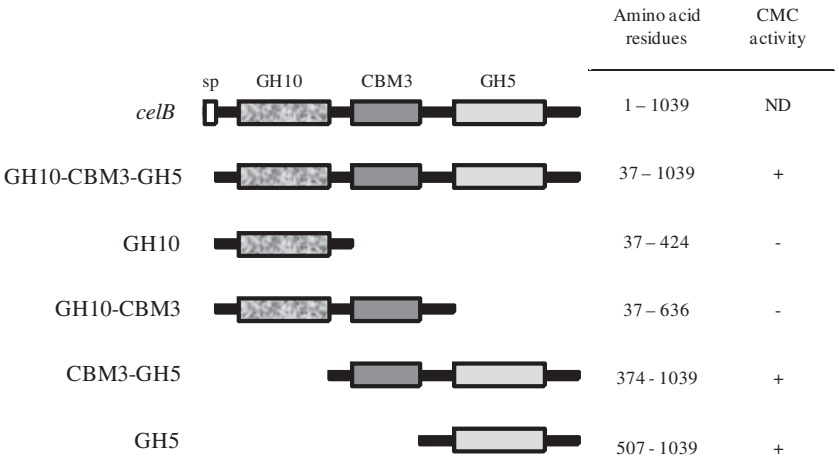

Fig. 1. The truncated constructs of the *celB* gene from *C. sac* were created in the pET DEST42 vector for protein expression in *E. coli*. The clear box represents the signal peptide (sp, aa 1–36), which was deleted in all truncated constructs tested in our study; the grained gray box represents GH10 (aa 44–373); light gray box represents GH5 (aa 636–975); and the dark gray box represents the CBM3 domain (aa 423–506). The boundaries of the putative domains were determined by Pfam database annotation. The activity of the expressed constructs was determined by zone-clearing assay using CMC. ND: not determined.

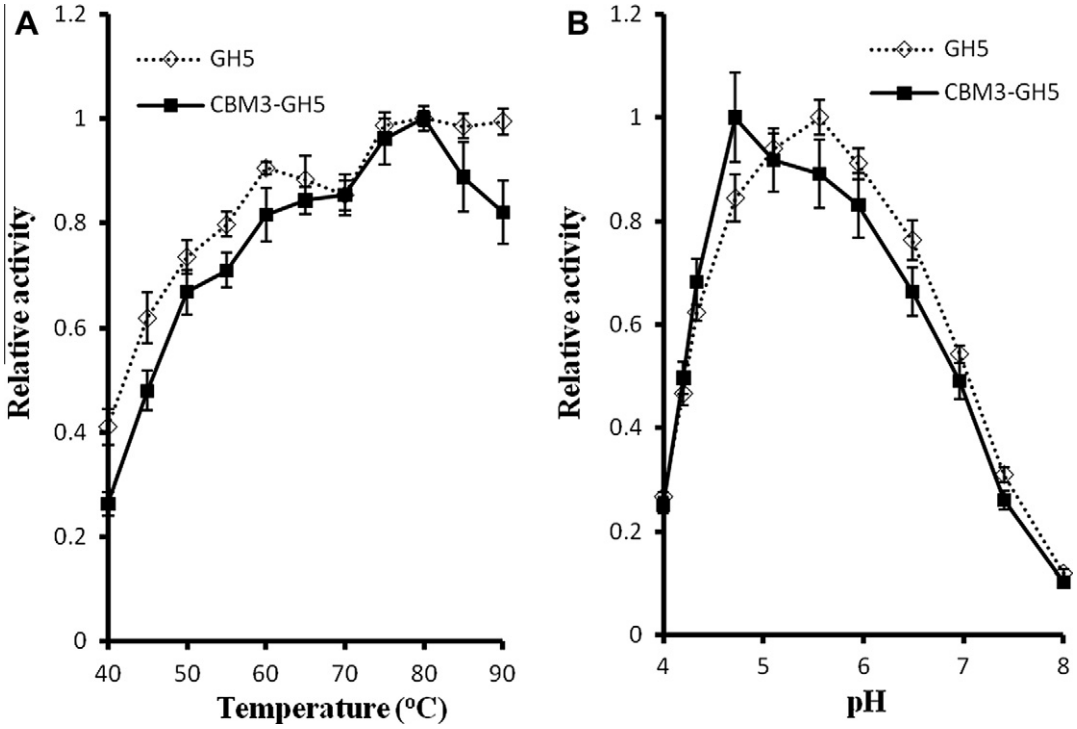

Fig. 2. Temperature (A) and pH (B)-dependent activity of CBM-GH5 and GH5 was measured by DNS assay using CMC.

expressed in 1 L cultures, and the recombinant proteins were purified by affinity (HisTrap FF) followed by ion-exchange (HiTrap Q) chromatography (Supplementary Fig. 3). The average yields for CBM3-GH5 and GH5 were 4 mg/L and 3 mg/L, respectively, as determined by Bradford assay.

3.2. Enzyme activity of CBM3-GH5 and GH5 on soluble substrates CMC, pNPC, and pNPG

After confirming that both CBM3-GH5 and GH5 hydrolyzed CMC, the optimum pH and temperature for the enzymatic activity of GH5 were determined using the DNS assay with CMC as the substrate. Based on the DNS assay results, the highest enzyme activity of CBM3-GH5 was observed at a pH of 4.7 and at 80 °C, while that of GH5 was observed at pH 5.5 and between 80 and 90 °C (Fig. 2). Thus, the T_{op} of enzymatic activity of CBM3-GH5 and GH5 are above the growth optimum of *C. sac* on cellulose, which is 70 °C at neutral pH (Rainey et al., 1994).

The function of GH5 was examined in detail using *p*NPC and *p*NPG assays to determine if GH5 has cellobiohydrolase and/or β-glucosidase activity, respectively. Both CBM3-GH5 and GH5 hydrolyzed *p*NPC, while neither constructs hydrolyzed *p*NPG up to one hour (data not shown). The kinetics of *p*NPC hydrolysis by CBM3-GH5 and GH5 were compared to see whether the CBM3 domain

influences the catalytic activity of GH5 on a soluble substrate. The kinetic parameters were obtained from four independent experiments. At pH 5.5 and 80 °C, CBM3-GH5 had a V_{max} of 0.62 ± 0.01 μmol/min/mg and a K_m of 2.4 ± 0.2 mM, whereas GH5 had a V_{max} of 0.71 ± 0.03 μmol/min/mg and a K_m of 2.2 ± 0.1 mM. The k_{cat} values were similar between CBM3-GH5 (49 ± 0.8 min^{-1}) and GH5 (46 ± 2 min^{-1}). Product inhibition by cellobiose was examined by adding unlabeled cellobiose into the reaction mixtures containing 5 mM of *p*NPC in 50 mM MES buffer at pH 5.5 and 80 °C. Under these conditions, cellobiose inhibited the catalytic activity of GH5 with an IC_{50} of 30 mM. These results indicate that the GH5 domain of *C. sac* is a cellobiohydrolase that lacks β-glucosidase activity and is inhibited by cellobiose. Furthermore, the CBM3 domain did not affect the kinetics of hydrolysis of the soluble substrate *p*NPC by the GH5 domain.

3.3. Thermostability of CBM3-GH5 and GH5

The thermostability of CBM3-GH5 and GH5 was measured by incubating the enzymes at 80 °C in 50 mM MES buffer at pH 5.5 (0.1 mg/mL protein concentration). At various time points of incubation, aliquots were taken and enzyme activity on *p*NPC was measured at 80 °C. The measured activity was normalized against activity at time zero. The half-life of GH5 (39 h) was longer than that of CBM3-GH5 (23 h) at 80 °C (Fig. 3A). At the optimum growth temperature of *C. sac* (70 °C), both CBM3-GH5 and GH5 retained more than 94% of activity after 48 h. After 96 h of incubation at 70 °C, CBM3-GH5 retained slightly more activity (77%) than GH5 (69%). At 95 °C, both proteins precipitated within 1 h and had no measurable activity. These results show superior thermostability of the recombinant CBM3-GH5 and GH5 at the optimum growth temperature of *C. sac* (70 °C) than at the optimum temperature of the enzymatic activity (80 °C).

Melting temperatures (T_m) of recombinant CBM3-GH5 and GH5 were measured by differential scanning calorimetry (DSC). The measured T_m of CBM3-GH5 was 80 °C, while that of GH5 was 89 °C (Fig. 3B). To examine whether the lower T_m of CBM3-GH5 is due to the presence of the CBM3 domain, we measured the T_m of the purified recombinant CBM3 (amino acid residues 374–635 of CelB) to be 79 °C; thus, it appears that the temperature dependant unfolding of CBM3-GH5 is driven by unfolding of the CBM3 domain, which in turn must destabilize the GH5 domain at this lower temperature. Thermal denaturation was irreversible for all of these recombinant proteins (data not shown).

3.4. Enzymatic hydrolysis of insoluble substrates by CBM3-GH5 and GH5 of C. sac

Having shown that GH5 hydrolyzes soluble substrates and that addition of CBM3 has only marginal effects on GH5 kinetics, we then tested the ability of GH5 and CBM3-GH5 to hydrolyze the solid substrates microcrystalline cellulose (Avicel), [C2mim][OAc]-pretreated Avicel, corn stover and [C2mim][OAc]-pretreated corn stover. Product analysis by HPAEC-PAD showed that cellobiose is the predominant product released from these solid substrates by CBM3-GH5 and GH5 (Fig. 4A, B, and D), except from the untreated corn stover, in which case neither GH5 nor CBM3-GH5 exhibited time-dependent sugar release (Fig. 4C). Interestingly, a small amount of glucose was also detected from the enzymatic hydrolysis of these solid substrates, although none of these recombinant proteins showed β-glucosidase activity by *p*NPG assay. The results of enzymatic hydrolysis experiments show that after 24 h incubation CBM3-GH5 releases 1.3-fold more cellobiose and 2-fold more glucose from [C2mim][OAc]-pretreated Avicel than does GH5 alone. Similarly, CBM3-GH5 releases 1.7-fold more cellobiose and 2.8-fold more glucose from [C2mim][OAc]-pretreated corn stover than does GH5 alone. On untreated Avicel, after 24 h CBM3-GH5 released 17% more cellobiose than did GH5 alone.

The experimental conditions designed for enzymatic hydrolysis of solid substrates were more favorable for GH5 than for

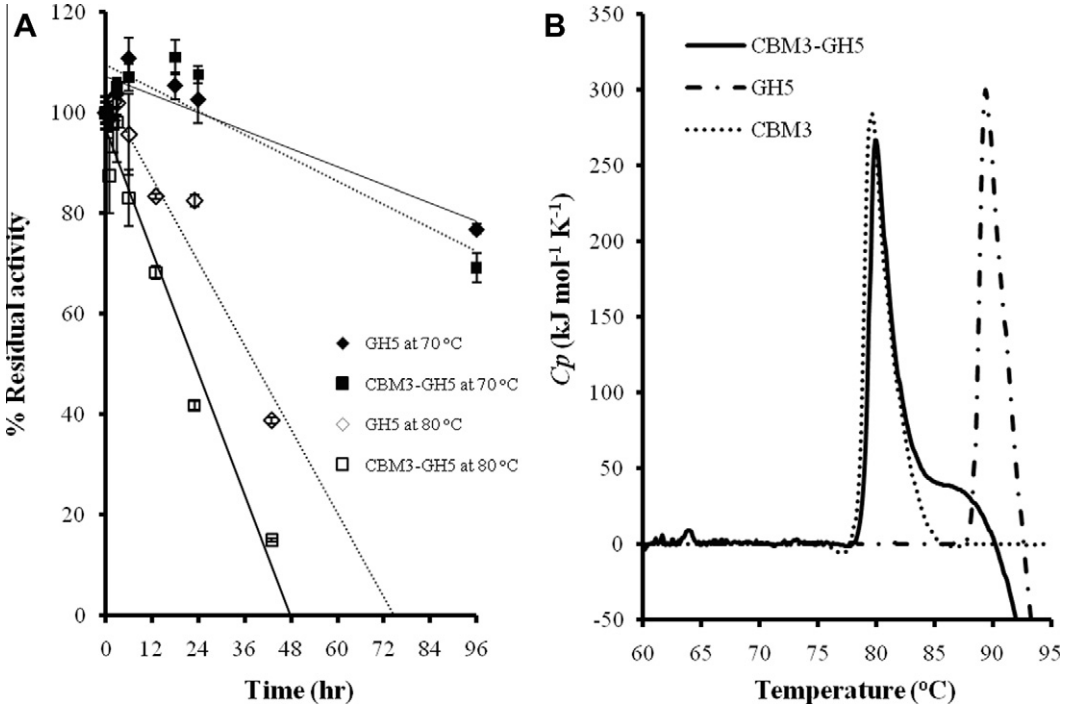

Fig. 3. Thermostability of recombinant CBM3-GH5 and GH5. A. Enzyme solutions containing CBM3-GH5 or GH5 were incubated at 70 °C and 80 °C. The enzyme activity on *p*NPC at each time point was normalized by the enzyme activity at the time zero, at the respective temperature of the corresponding construct. The error bars represent standard deviations. Based on linear regression analysis, half-lives of CBM3-GH5 and GH5 at 80 °C were 22 h and 32 h, respectively. At 70 °C, at least 74% of residual activity remained for both CBM3-GH5 and GH5 after 96 h. B: Melting temperature (T_m) measurements of recombinant CBM3-GH5 (80 °C), GH5 (89 °C), and CBM3 (79 °C).

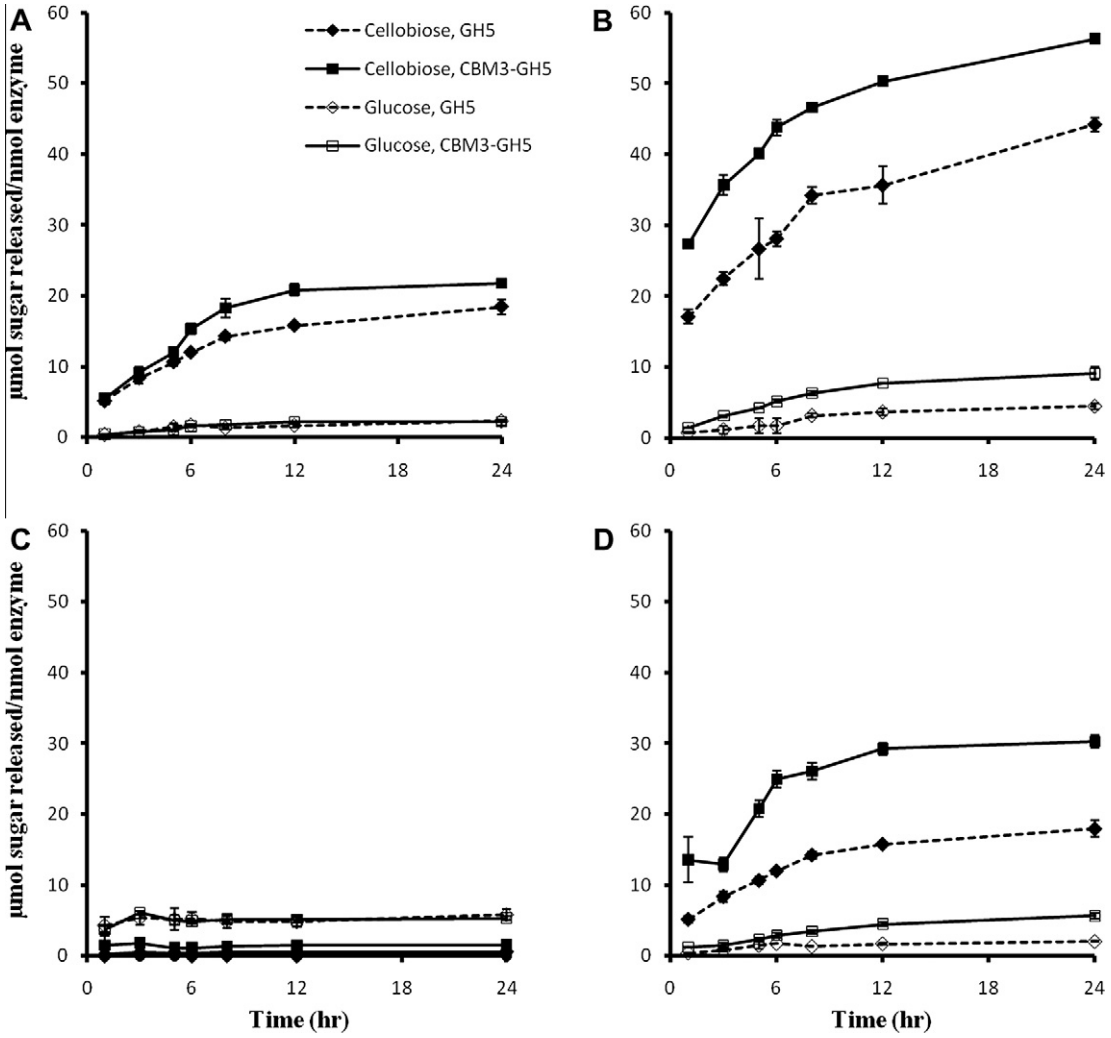

Fig. 4. Enzymatic hydrolysis of solid substrates by recombinant CBM3-GH5 and GH5 as a function of time. The enzymes and substrates were incubated at 80 °C in 50 mM MES buffer at pH 5.5. A: microcrystalline Avicel, B: [C2mim][OAc]-pretreated Avicel, C: corn stover (untreated), and D: [C2mim][OAc]-pretreated corn stover.

CBM3-GH5, since the half-life of GH5 is longer than that of CBM3-GH5 at 80 °C (Fig. 3A). The results clearly show that the CBM3 domain improved the catalytic activity of the attached GH5 domain on these substrates, despite the experimental findings that CBM3-GH5 had a lower half-life of enzymatic activity and a lower T_m than GH5. Low levels of glucose were released from Avicel and corn stover hydrolyzed by CBM3-GH5 and GH5, even though β-glucosidase activity was not detected by using *p*NPG. Analysis of oligosaccharides from the enzymatic digestion of Avicel on HPLC showed cellobiose as the major product, and trace amounts of cellotriose and glucose (data not shown). Therefore, it is possible that glucose produced in the solid substrate hydrolysis reactions was from the hydrolysis of cellotriose to cellobiose and glucose or from the hydrolysis of polysaccharides with $DP = n$ to glucose and a polysaccharide with $DP = n - 1$.

Cellulases that hydrolyze insoluble cellulose often contain accessory domains such as CBMs that play important roles in substrate recognition and binding and enzymatic degradation of cellulose (Boraston et al., 2004; Shoseyov et al., 2006). CelJ from the thermophilic bacterium *Clostridium thermocellum* contains CBM30 and GH9 domains (Ahsan et al., 1996). Interestingly, while the GH9 domain alone had negligible activity on Avicel and CMC, addition of CBM30 to GH9 restored the ability of GH9 to hydrolyze both of these substrates (Arai et al., 2003). Furthermore, enhanced enzymatic activity of an endoglucanase from the hyperthermophilic archaea *Pyrococcus horikoshii* on Avicel and CMC was observed when the CBM2 domain (a chitin-binding domain) from *Pyrococcus furiosus* was fused to the enzyme (Kang et al., 2007). This study on CelB of *C. sac* shows that the non-catalytic CBM3 enhances hydrolytic activity of the linked GH5 on [C2mim][OAc]-pretreated Avicel and corn stover, although the kinetics of enzyme activity of GH5 on hydrolysis of *p*NPC was unaffected by CBM3 (Section 3.2).

3.5. Binding interactions between CBM3-GH5 or GH5 and cellulose

Confocal imaging was used to examine the substrate binding characteristics of the fluorophore labeled CBM3-GH5, GH5, and CBM3 (Supplementary Fig. 4). The data indicate that in all cases there is a measurable amount of protein bound to the substrate after incubating for 30 min at 70 °C followed by extensive rinsing with buffer. Since the number of dye molecules per protein was held constant at 0.9 ± 0.1 for GH5, CBM3, and GH5-CBM3, the relative fluorescence intensity levels reflect the relative number of protein molecules remaining on each substrate after rinsing. The intensities for both substrates were measured for the same 1 μm thickness defined by the confocal geometry. The binding results show that on [C2mim][OAc]-pretreated Avicel the amount of tightly bound CBM3-GH5 is 12-fold higher than bound GH5 alone

and 3.5-fold higher than bound CBM3, whereas on untreated Avicel the amount of tightly-bound CBM3-GH5 is comparable to the amounts of bound GH5 and CBM3 (Supplementary Fig. 4).

The present work indicates a correlation between enzyme activity and the amount of irreversibly bound protein. The results show a much greater increase in bound protein for CBM3-GH5 relative to GH5 on [C2mim][OAc]-pretreated Avicel than on Avicel. Correspondingly, Fig. 4 shows that the activity enhancement for CBM3-GH5 relative to GH5 is greater on [C2mim][OAc]-pretreated Avicel than on Avicel. This suggests that the amorphous nature of [C2mim][OAc]-pretreated Avicel promotes stronger binding for CBM3-GH5 than for GH5 alone, presumably by exposing a larger number of properly oriented CBM3 binding sites. The higher level of binding that results when CBM3 is combined with GH5 enhances the activity, perhaps through holding the GH5 domain in close contact with the substrate for a longer period of time upon each encounter, allowing multiple hydrolysis events for each binding event.

4. Conclusions

This study shows that the previously uncharacterized GH5 domain of CelB from *C. sac* is a cellobiohydrolase with optimum activity at pH 4.7-5.5 and 80 °C. GH5 activity on Avicel and corn stover was enhanced upon pretreatment with [C2mim][OAc], and activity was further improved when the CBM3 domain was fused to GH5. This enhancement of enzymatic activity is attributed to the synergistic effect of binding to solid substrates by fusing the CBM3 to the GH5. These recombinant enzymes isolated from *C. sac* are suitable biocatalysts for liberating cellobiose from [C2mim][OAc] pretreated biomass at high temperatures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.02.036.

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